

## SHORT REPORT: DETECTION OF *ORIENTIA TSUTSUGAMUSHI* IN CLINICAL SAMPLES BY QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

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**Abstract.** *Orientia tsutsugamushi* infection causes scrub typhus, a common zoonosis of rural Asia. *Orientia tsutsugamushi* was recently detected by a real-time quantitative polymerase chain reaction (qPCR) assay in animal specimens. We evaluated the same qPCR assay in specimens obtained from patients with serologically proven scrub typhus infections. The 47-kDa qPCR assay was more sensitive than was mouse inoculation; it was reactive in whole blood specimens from all 10 isolate-positive patients and in 7 of 17 isolate-negative individuals ( $P = 0.003$ , Fisher's two-tailed exact test). As few as 1,076 *O. tsutsugamushi* copies/ $\mu\text{L}$  were detected in whole blood. Four of 7 sera from isolate-proven scrub typhus infections were also reactive by qPCR. The assay was unreactive in all 12 individuals without scrub typhus infection. This is the first demonstration of a sensitive and specific real-time qPCR assay for human scrub typhus infection.

Scrub typhus is an acute, febrile zoonosis caused by the obligate intracellular bacterium *Orientia tsutsugamushi*. The disease is of greatest public health importance in rural areas of Asia and on Western Pacific Islands. Prompt, accurate diagnosis allows institution of specific antibiotic treatment, which markedly reduces morbidity and mortality.<sup>1,2</sup> Effective serologic assays have been developed that use crude *O. tsutsugamushi* antigens<sup>3</sup> and, more recently, recombinant 56-kDa outer membrane protein antigens.<sup>4</sup> *Orientia tsutsugamushi* can be isolated by inoculating patient blood into mice, but results are not available in time to guide clinical management. The causative organism can also be demonstrated by standard and by nested PCR.<sup>5–7</sup> Real-time PCR assays are as sensitive as standard PCR but are more rapid and can give quantitative results. A real-time quantitative PCR assay (qPCR) for detecting *O. tsutsugamushi* nucleic acid in infected mouse and monkey blood was recently described.<sup>8</sup> Using the same primer and probe as did Jiang and others, we performed qPCR on clinical specimens taken from Thai patients with proven scrub typhus infection. The primer was designed based on sequences of an *O. tsutsugamushi* 47-kDa surface antigen.<sup>8</sup> The 47-kDa gene sequences of the Karp, Kato, Gilliam, and Boryong strains of *O. tsutsugamushi* are similar to those of the genes of the high-temperature requirement (HtrA) family of stress response proteins that have both chaperone and endoprotease activities.<sup>8</sup>

Whole blood and sera had originally been collected during a treatment trial of drug-resistant scrub typhus in Chiangrai, northern Thailand.<sup>9</sup> *Orientia tsutsugamushi* infection was confirmed serologically by the indirect immunoperoxidase test when the IgM titer on admission was 1:400 or greater and/or the IgG titer was 1:1600 or greater. *Orientia tsutsugamushi* was isolated from some patients using animal passage. Briefly, ICR mice were inoculated intraperitoneally with 0.2 mL of whole patient blood at the bedside. After several passages, organisms were demonstrated in liver-spleen homogenates by Giemsa stain.<sup>9</sup> Control specimens from individuals without scrub typhus were taken from normal volunteers who lived in areas not endemic for *O. tsutsugamushi* and from patients with dengue fever and leptospirosis. Genomic DNA from 200  $\mu\text{L}$  human whole blood and 200  $\mu\text{L}$  human sera was extracted

using QIAamp DNA Blood Mini Kits (Qiagen, Hilden, Germany).

We used LightCycler (Idaho Technology, Salt Lake City, UT, USA) with paired oligonucleotide primers specific for the Rickettsia amplicon for qPCR. A 20  $\mu\text{L}$  total volume sample mixture was used that included 10  $\mu\text{L}$  of DNA template, 2.0  $\mu\text{L}$  of a LightCycler FastStart DNA Hybridization Probe kit (Roche Applied Science, Penzberg, Germany) that included Taq DNA polymerase and reaction buffer dNTP mix, and 2.4  $\mu\text{L}$  of 3 mM  $\text{MgCl}_2$  (Roche Applied Science, Penzberg, Germany). Also used were 0.1  $\mu\text{M}$  of forward and reverse primers (O.tsu630F and O.tsu 747R)<sup>8</sup> and 0.2  $\mu\text{M}$  O.tsuPR665 TaqMan probe.<sup>8</sup> Specimens for PCR were incubated at 94°C for 3 minutes, followed by 80 cycles of two-step amplification at 94°C for 5 seconds and combined annealing/extension at 60°C for 30 seconds. The temperature transition rate was set at 20°C per second. Normal DNA was used as a negative control, and L929 mouse fibroblast cells infected by *O. tsutsugamushi* were used as a positive control. Plasmids containing a single copy of the Kato 47-kDa gene and diluted from  $10^{10}$  to  $10^0$  using AE buffer were used for qPCR using multiple beads (OmniMix HS, TaKaRa Bio Inc., Japan).

DNA specimens from patients without scrub typhus were used as negative controls. All 12 such DNA specimens were unreactive by real-time PCR. These included nine normal volunteers, two patients with dengue fever, and one patient with leptospirosis. Positive controls included plasmid DNA and DNA from *O. tsutsugamushi*-infected cell culture. All positive control specimens were reactive by real-time PCR. A total of 27 specimens from patients with serologically proven scrub typhus infection were assayed; 10 specimens were from patients in whom *O. tsutsugamushi* had been demonstrated by mouse inoculation, and 17 specimens were from patients in whom mouse inoculation had not demonstrated *O. tsutsugamushi*. PCR was more sensitive for detecting *O. tsutsugamushi* than was mouse inoculation (Table 1). All 10 isolate-positive specimens were reactive by PCR, as were 7 of 17 isolate-negative specimens ( $P = 0.003$ , two-tailed Fisher's exact test). Seven sera from mouse isolate-positive patients were also tested by PCR; 4 of the 7 were reactive.

Seven plasmid dilutions from  $10^6$  to  $10^0$  copies were made

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TABLE 1

Detection of *Orientia tsutsugamushi* by real-time polymerase chain reaction (PCR)\*

	PCR positive	PCR negative
Isolate positive	10	0
Isolate negative	7	10

\* Twenty-seven whole blood specimens from patients with serologically proven scrub typhus were assayed. Ten specimens were from patients in whom *O. tsutsugamushi* was demonstrated by mouse inoculation (isolate positive), and 17 specimens were from patients in whom the organism was not demonstrated (isolate negative).

to perform qPCR. Neither  $10^1$  nor  $10^0$  plasmid copies could be detected. qPCR was performed on eight whole blood specimens that had previously been real-time PCR-reactive and on four that had previously been unreactive. All four previously unreactive specimens were again unreactive, as was one specimen that had previously been reactive. For the seven reactive samples, copy numbers were similar in mouse isolate-positive and mouse isolate-negative specimens (Table 2).

*Orientia tsutsugamushi* has previously been demonstrated in human specimens by both standard and nested PCR.<sup>5-7</sup> Our study is the first description of the use of real-time PCR to detect *O. tsutsugamushi* in human specimens. The *O. tsutsugamushi* 47-kDa PCR assay was sensitive and specific for the detection and enumeration of scrub typhus DNA in human specimens, as had been the case for monkey and mouse samples.<sup>8</sup> Indeed, organisms could be demonstrated by PCR in samples from which *O. tsutsugamushi* could not be isolated by mouse inoculation (Table 1). *Orientia tsutsugamushi* was also demonstrated by PCR in acute sera from four of seven isolate-positive individuals. *Orientia tsutsugamushi* is intracellular, so performing qPCR on sera would be expected to be less sensitive. However, performing this assay on sera might be a useful option when cellular specimens are not available.

There are several applications of qPCR for scrub typhus, although its use for routine diagnosis is probably not necessary because several excellent, less expensive, rapid antibody-based serological tests have been developed.<sup>4</sup> Rapid tests are positive in more than 90% of individuals with scrub typhus during the first week of fever.<sup>3</sup> However, relapsing scrub typhus cannot be distinguished from other causes of fever by serology. The demonstration of *O. tsutsugamushi* by qPCR would be very helpful for the diagnosis of relapse. Real-time PCR could also be useful in cases where serology results are not clear-cut. The demonstration of scrub typhus with diminished antibiotic susceptibility<sup>10</sup> has stimulated a search for antibiotics more effective than tetracycline and chloramphenicol. Quantitative PCR could serve as an objective marker of treatment response in clinical trials of new antibiotics for the treatment of *O. tsutsugamushi* infection.

TABLE 2

Copy numbers of *O. tsutsugamushi* 47-kDa gene/ $\mu$ L of blood in 8 patients with serologically proven scrub typhus\*

Copies of <i>Orientia tsutsugamushi</i> 47-kDa gene/ $\mu$ L	
Isolate positive	Isolate negative
1. 28,810	5. Unreactive
2. 21,040	6. 23,790
3. 1,076	7. 1,298
4. 4,232	8. 4,265

\* Four specimens were from patients in whom *O. tsutsugamushi* had been demonstrated by mouse inoculation (isolate positive, specimens 1-4) and four specimens were from patients in whom organisms had not been demonstrated by mouse inoculation (isolate negative, specimens 5-8).

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## REFERENCES

- Watt G, Parola P, 2003. Scrub typhus and tropical rickettsioses. *Curr Opin Infect Dis* 16: 429-436.
- Rosenberg R, 1997. Drug resistant scrub typhus: paradigm and paradox. *Parasitol Today* 13: 131-132.
- Watt G, Strickman D, Kantipong P, Jongsakul K, Paxton H, 1998. Performance of a dot blot immunoassay for the rapid diagnosis of scrub typhus in a longitudinal case series. *J Infect Dis* 177: 800-802.
- Coleman RE, Sangkasuwan V, Suwanabun N, Ching W-M, Sattabongkot J, Eamsila C, Richards AL, Rowland D, Devine P, Lerthusnee K, 2002. Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to *Orientia tsutsugamushi* in Thailand. *Am J Trop Med Hyg* 67: 497-503.
- Murai K, Tachibana N, Okayama A, Shishime E, Tsuda K, Oshikawa T, 1992. Sensitivity of polymerase chain reaction assay for *Rickettsia tsutsugamushi* in patients' blood samples. *Microbiol Immunol* 36: 1145-1153.
- Manosroi J, Chitipongvivate S, Auwanit W, Manosroi A, 2003. Early diagnosis of scrub typhus in Thailand from clinical specimens by nested polymerase chain reaction. *Southeast Asian J Trop Med Public Health* 34: 831-838.
- Saisongkorh W, Chenchittikul M, Silpapojakul K, 2004. Evaluation of nested PCR for the diagnosis of scrub typhus among patients with acute pyrexia of unknown origin. *Trans Roy Soc Trop Med Hyg* 98: 360-366.
- Jiang J, Chan T-C, Temenak JJ, Dasch GA, Ching W-M, Richards AL, 2004. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. *Am J Trop Med Hyg* 70: 351-356.
- Watt G, Kantipong P, Jongsakul K, Watcharapichat P, Phulsuksombati D, Strickman D, 2000. Doxycycline and rifampicin for mild scrub-typhus infections in northern Thailand: a randomized trial. *Lancet* 356: 1057-1061.
- Watt G, Chouriyagune C, Ruangwearayud R, Watcharapichat P, Phulsuksombati D, Jongsakul K, Teja-Isavadharm P, Bhodhidatta D, Corcoran KD, Dasch GA, Strickman D, 1996. Scrub typhus infections poorly responsive to antibiotics in northern Thailand. *Lancet* 348: 86-89.